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# CYTOTOXIC EFFECT OF GREEN SYNTHESIZED SILVER NANOPARTICLES USING *Indigofera longeracemosa* ON SKIN CANCER SK MEL-28 CELL LINES

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**ABSTRACT**

The present study is focused on the biosynthesis of stable silver nanoparticles (AgNPs) from *Indigofera longeracemosa* and its anticancer effects in human skin cancer skin cell line SK-MEL-28 was investigated. The AgNPs synthesis was determined by UV- visible spectrum and it was further characterized by FT-IR, Scanning Electron Microscopy (SEM), and X-Ray Diffraction (XRD) analysis. The cytotoxicity of the nanoparticles was determined against SK-MEL-28 cell line at different concentrations by MTT assay. Cell morphology and DNA fragmentation were also analyzed using AgNps of *Indigofera longeracemosa*. Also since tumorigenesis is thought to result from a series of progressive gene alterations, including activation of oncogenes and inactivation of tumour suppressor genes, expression of two such genes, P53 and Bcl-2 that are believed to play a crucial role in tumourigenesis was determined. UV visible spectrum indicated an absorption peak at 436 nm which reflects its specific Surface Plasmon Resonance (SPR). Biosynthesized AgNPs were predominantly spherical and appeared in the size ranging from 30nm to 110nm as observed through SEM. Cytotoxicity of biosynthesized AgNPs against *in vitro* Human skin cancer cell line SK MEL-28 showed a dose-response activity with an $IC_{50}$ value of 48µg/ml. DNA fragmentation assay confirmed induction of apoptosis in SK-MEL-28 cells treated with AgNps of *Indigofera longeracemosa*. Apoptotic cells were characterized by condensed and fragmented DNA. From RT-PCR analysis it was clear that, AgNPs of *Indigofera longeracemosa* decreased the expression of anti apoptotic gene Bcl-2 significantly and increased the expression of tumour suppressor gene p53. Since the AgNPs of *Indigofera longeracemosa* observed to be most effective in cell growth inhibition and induction of apoptosis in SK-MEL-28 cells, it can be considered as effective cytotoxic agent. Further toxicity studies of AgNPs of *Indigofera longeracemosa* on animal models might open a door for a range of anticancer agents.

**Key Words**: *Indigofera longeracemosa*, SK MEL-28 cell line, Anticancer activity, Silver nano particles, XRD, SEM.

## INTRODUCTION

Cancer is a class of diseases in which a cell or a group of cells display uncontrolled growth, invasion and metastasis [1]. Cancer chemoprevention applies specific natural or synthetic chemical compounds to inhibit or reverse carcinogenesis and to suppress the development of cancer from premalignant lesions [2]. A major problem with present cancer chemotherapy is the serious deficiency of active drugs for the curative therapy of tumours [3]. Because of high death rate associated with cancer and because of the serious side effects of chemotherapy and radiation therapy, many cancer patients seek alternative and/or complementary methods of treatment [4]. Silver nanoparticles (AgNPs) have a great potential in cancer management because it selectively involved disruption of the mitochondrial respiratory chain by AgNPs leading to production of ROS and interruption of ATP synthesis, which in turn cause DNA damage [5, 6]. Since nanoparticles (NPs) are more biocompatible than the conventional therapeutics, they are exploited for drug encapsulation and delivery [7].

Cutaneous melanoma is one of the most aggressive forms of skin cancer with high metastatic potential and strong resistance to radiation, immunotherapy

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and chemotherapy [8]**.** Melanoma is the eighth most commonly diagnosed disease in the USA [9] and its incidence is rising world-wide at a rate of about 5% per year [10]. Chemo-therapy of late diagnosed and metastatic melanoma cases with dacarbazine and alkylating agents has important side effects. Moreover, melanoma has one of the worst rates of response to chemotherapy of all neoplasias [11]. These problems have led to the search for new types of treatment [12] and for new compounds with fewer side effects. Recent studies, however, have indicated that melanoma cells show dysfunction in the apoptotic program [13] which provided exciting new targets for rationally designed anti-melanoma therapeutic strategies. Wild type $P^{53}$ is an important regulatory protein in induction of apoptosis following DNA damage induced by anti cancer drugs [14,15] . The tumor suppressor gene p53 is regarded as the master guardian of the cell and is able to activate cell-cycle checkpoints, DNA repair, and apoptosis to maintain genomic stability [16] is reported to induce apoptosis cell death by direct or indirect modulating expression of Bcl-2 family of proteins Bcl-2 and Bax [17]. The bax/bcl-2 protein ratio determines the life or death of cells in response to an apoptotic stimulus. A higher level of bax/bcl-2 ratio decreases the resistance to apoptotic stimuli, leading to apoptosis [18]. The Bcl-2 itself is an anti-apoptotic gene that prevents initiation steps of apoptosis and programmed cell death [19] .

Many plant-derived products have been reported to exhibit potent antitumour activity against several rodent and human cancer cell lines [20]. *Indigofera longeracemosa* a tropical shrub plant belonging to the family Fabaceae has been used as a diuretic. In India, the root has also been used in tribal medicine as an antidote for all snake poison [21]. Many compounds with antimicrobial, antiulcerogenic, pharmacognostical activities have been isolated from Indigofera longeracemosa [22, 23]. The chemical composition of the leaves of Indigofera longeracemosa were investigated but cytotoxicity and anticancer activities of this plant have not been reported previously. Therefore the main aim of this study was to synthesize, characterize silver nano particles from Indigofera longeracemosa and to evaluate their cytotoxic properties and antiproliferative effect on skin cancer cell lines. The expression of P53 and Bcl-2 genes was also determined to study the molecular mechanisms of anticancer effects of AgNps on skin cancer cell lines.

## MATERIALS AND METHODS

### Preparation of leaf aqueous extract

Fresh plant, *I.longeracemosa* was collected from Kozhijampara and authenticated from Botanical survey of India, Tamil Nadu Agricultural University, Coimbatore, India. Fresh leaves were used for the extraction of the active components. The leaves were shade dried for 2 to 3 weeks at room temperature and then powdered. Briefly, 5 g of leaf powder was weighed and mixed in 100 mL of millipore water. This mixture was boiled in water bath at 60°C for 10- 20 min. After cooling to room temperature, the mixture was filtered by using muslin filter cloth (0.2 µm) and used for the study.

### Green Synthesis of AgNPs

Biological synthesis of AgNPs was carried out following Song *et al.* [24] method. Typically, 10 mL of aqueous extract was added to 190 mL of 1 mM aqueous silver nitrate solution for the reduction of Ag+ ions. The AgNPs solution, thus obtained was purified by repeating the centrifugation thrice at 7,000 rpm for 20 min at 4°C followed by redispersion of the pellet in Milli-Q water.

### Characterization of AgNPs

UV–visible spectra were recorded as a function of the reaction time on a UV- visible ELICO SL 159 nanodrop spectrophotometer at a resolution of 1 nm. The purified AgNPs were examined for the presence of biomolecules using FTIR analysis. The spectrum obtained from the dried sample was recorded on FTIR spectrum RX-1 instrument (Perkin- Elmer), USA in the diffuse reflectance mode at a resolution of 4 $cm^{-1}$ in KBr pellets. Crystalline AgNPs were determined by X-ray diffraction analysis using X-ray diffraction instrument (PXRD-6000 SCIMADZU) in the angle range of $10^0$C-$80^0$C.Briefly, the biosynthesized AgNPs were laid onto the glass substrates on a Phillips PW 1830 instrument operating at a voltage of 40 kV and a current of 30 mA with Cu*Kα* radiation. The shape of the freeze dried AgNPs was analyzed by SEM (SEM- Hitachi model- S 3000H).

### In vitro cytotoxicity of biosynthesized AgNPs

SK-Mel-28 cell lines were purchased from National Centre for Cell Science (NCCS), Pune and maintained in Dulbecco's modified eagles media, supplemented with calf serum (10%) and non-essential amino acids. Cells were cultured at 37°C in a humidified atmosphere containing 5 % CO2 in air and seeded into 96 well plates approximately as 1 x $10^4$ cells in each plate and incubated for 48 hrs. AgNPs of *Indigofera longeracemosa* were added to grown cells at a final concentration of 10µg, 50µg and 100µg/ml from a stock of 10mg/ml and also standard drug (Sorafenib) was added in the same concentration and incubated for 24 hours. The percentage difference in viability was determined by MTT assay [25] after 24 hours of incubation.

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a yellow tetrazole) was prepared in 5 mg/ml concentration. 100 µl of MTT was added in each well and incubated for 4hrs Purple color formazone crystals formed were then dissolved with 100 µl of Di-methyl sulphoxide (DMSO). These crystals were observed at 620 nm in a Multi well ELISA plate reader. OD value was subjected to sort out percentage of viability by using the following

formula, Percentage of viability=

$$\frac{\text{OD value of experimental samples (AgNPs)}}{\text{OD value of experimental control (untreated)}} \times 100$$

**Morphological Analysis**

Human skin cancer cells were grown to 70% confluence and treated with defined concentration (10, 50 and 100µg/ml) of AgNPs of *Indigofera longeracemosa* for 24h and the photographs were taken at 40X magnification using a phase-contrast inverse microscope (Olympus, Japan).

**DNA Fragmentation Assay**

Fragmentation of chromatin to units of single or multiple nucleosomes that form the nucleosomal DNA ladder in agarose gel is an established hallmark of programmed cell death or apoptosis. The cells grown to about 70% confluence and treated with defined concentration (10, 50 and 100µg/ml) of AgNPs of *Indigofera longeracemosa* for 48hrs and subjected to processing for DNA isolation and fragmentation assay as previously described [26,27]. The bands were visualized under an UV transilluminator, followed by digital photography.

**RT-PCR**

The mRNA expression levels of p53 and Bcl-2 carried out using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Briefly, the SKMEL-28 cells were cultured in T-25 flasks and maintained in Dulbecco's modified eagles medium for 48 hrs. The Dulbecco's modified eagles medium was supplemented with FBS and pen-strep. To the flask was added the required concentration of AgNPs of *Indigofera longeracemosa* to inhibit 50% growth and incubated for 24 hrs. Total cellular RNA was isolated from the untreated (control flask) and treated cells using Tri Reagent according to manufacturer's protocol. cDNA was synthesized from 2µg of total isolated RNA by incubation for 1hr at 42°C with M-MLV reverse transcriptase (Fermentas) and oligo(dT)18 primer according to the manufacturer's instruction. Then 2.5µl of the reaction mixture was subjected to PCR for amplification of p53 and Bcl-2 cDNAs using specifically designed primers by BLAST analysis of Genebank sequences available for these genes (p53 Forward: 5′-C T G A G G T T G G C T C T G A C T G T A C C A C C A T C C-3′; p53 Reverse: 5′-C T C A T T C A G C T C T C G G A A C A T C T C G A A G C G-3′ and Bcl-2 Forward: 5′-G T T C G G T G G G G T C A T G T G T G T G G A G A-3′; Bcl-2 Reverse: 5′-G C T G A T T C G A C G T T T T G C C T G A A G A C-3′). As an internal control, the house keeping gene GAPDH (GAPDH Forward:5′-CTCAGAA GACTGTG GATGG-3′;GADPH Reverse: 5′-GTCATCA TACTTGG CAGGTT-3′) was co-amplified in each reaction. The PCR reactions was carried out in a final volume of 50 µl containing 1x PCR buffer and 5 U/µl Taq-polymerase (Fermentas), 1.5 mM MgCl2 , 0.2mM of each dNTP and 0.4 µM of each primer. The template was denatured for 5min at 94°C, followed by amplification cycles at 94°C for 1min, 69°C (for p53, 64°C for Bcl-2 and 57°C for GADPH) for 1min and 72°C for 1.20 min, and terminated with an additional extension step for 8 min at 72°C. The PCR products were visualized using 1.2% agarose gel electrophoresis with ethidium bromide staining. In negative control, template cDNA was replaced by DEPC water.

**Statistical analysis**

The significance between the control and treated groups was determined by Student's t test, and p values of less than 0.05 were taken to be significant in the experiments.

**RESULTS AND DISCUSSION**

**Visual Characterisation**

As the aqueous *Indigofera longeracemosa* extract was mixed with aqueous solution of 1 mM silver nitrate, it started to change colour from colourless to reddish brown due to reduction of silver ions; which indicates the formation of silver nanoparticles (Fig.1).

**Characterisation of Biosynthesized Silver Nanoparticles by spectral methods**

**UV-Visible Spectroscopy**

The formation of silver nanoparticles was confirmed by color changes followed by UV–Visible spectrophotometer analysis. The UV–Visible spectrophotometer has proved to be a very useful technique for the analysis of some metal nanoparticles and is a significant technique to authenticate the formation and stability of AgNPs in aqueous solution. The reduction of the pure Ag+ ions was monitored by measuring the UV-Vis spectrum of the reaction medium at 5 hours (complete colour change) following the dilution of a small aliquot of the sample in distilled water. The UV-Vis spectral analysis was conducted using ELICO SL 159 nanodropspectrophotometer. The reduction of silver ions in the aqueous solution of nanoparticles in the solution could be correlated with the respective UV-Vis Spectra of the colloidal solution which exhibited a strong absorption at 360nm as shown in Fig. 2.

**FT-IR**

FTIR spectroscopy is useful in probing the chemical composition of the surface of the silver nanoparticles and the local molecular environment of the capping agents on the nanoparticles. The FTIR spectrum of silver nanopowder is shown in Figure 3. The broad band appearing at 3241 $cm^{-1}$ is assigned for O−H stretching vibration indicating the presence of hydroxyl groups in the reducing agent . The band at 2845 $cm^{-1}$ correspond to C-

H stretching from methyl or methylene groups. The absorption band at around 1408-1424.57, 1519.55-1535.66 correspond to cyclohexane CH=CH stretching and C-C deformation. 1188.65 shows the presence of c-o stretching of ketone or ester. The IR spectrum of the AgNPs indicates the absence of many fundamental groups and peaks of lower intensity. The disappearance of the bands and the decrease in intensity is attributed to reduction of silver ions.

**XRD**

XRD is commonly used for determining the chemical composition and crystal structure of a material; therefore, detecting the presence of silver nanoparticles in plant tissues can be achieved by using XRD by examining the diffraction peaks of the plant. The crystalline nature of Ag nanoparticles was also confirmed from X-ray diffraction (XRD) analysis. The sharp diffraction patterns of the XRD spectra obtained by annealing at $200^0$C indicating a pure crystalline silver structure. Figure 4 shows four peaks observed at 2θ values of 34.250, $37.77^0$, $48.15^0$, $62.25^0$ corresponding to the planes of silver respectively. So the structure is Orthorhombic and the X-rays were scattered by diffraction owing to the unique crystalline structure of the material analyzed. The observed peak broadening and noise were probably macromolecules present in the plant extract which may be responsible for the reduction of silver ions. Hence XRD pattern thus clearly illustrated that the silver nanoparticles formed in this present synthesis are crystalline in nature.

**Morphological studies of silver nanoparticles by using Scanning Electron Microscopy (SEM)**

A SEM employed to analyze the morphology and size details of the silver nanoparticles that were formed. From (Fig. 5**)** it was showed that the silver nanoparticles formed were spherical in shape, with the size ranging from 30nm to 110nm and uniformly distributed silver nanoparticles on the surface of the cells were observed. A similar phenomenon has been reported [28, 29].

**Determination invitro anticancer Effect of AgNPs of *Indigofera longeracemosa* on cultured SKMEL 28 cell lines**

The *invitro* cytotoxicity of the AgNPs of *Indigofera longeracemosa* was evaluated against Human skin cancer cell lines SK MEL-28 cell line at different concentrations( Table.No.1). The samples demonstrated a considerable cytotoxicity against the SK MEL-28 cell line . The result showed that SK MEL-28 cell proliferation was significantly inhibited by AgNPs with an $IC_{50}$ value of 48 µg/ml of the concentration. The % toxicity increases with increase in concentration of silver nano particles suggests that biosynthesized silver nanoparticles could be of immense use in medical field to certain extent as anticancer agent (Fig.6). From the results indicated in Table 1 it is seen that percentage viability decreases with concentration whereas cytotoxicity increases with concentration demonstrating a direct dose dependent relationship. The results were correlated with the findings of Mercy ranjitham *et al.* [30] who have reported increased cytotoxicity of silver nanoparticles synthesized from aqueous extract of fresh Cauliflower floret on MCF-7 breast cancer cell line in a dose dependent manner.

The cytotoxic effect of AgNps on cell viability has a major role in anticancer activity thereby reducing disease progression. The cytotoxic effects of silver were the results of active physiochemical interaction of silver atoms with the functional group of intra cellular proteins as well as with the nitrogen bases and phosphate groups in DNA. The DNA fragmentation experiments subsequently confirmed induction of apoptosis.

**Morphological Analysis**

Apoptosis was investigated by molecular and morphologic approaches. The ultrastructural analysis by light microscope was performed to describe the apoptosis of SK-MEL-28 cells. When AgNPS of *Indigofera longeracemosa* at different concentrations (10, 50 and 100µg/ml) was added to SKMEL 28 cells, the cells showed typical apoptosis characterized by chromatin condensation close to the nuclear envelope, associated with micronuclei formation and cytoplasmic vacuolization. Our results were in accordance with Akbar *et al.* [31] who have reported typical features of apoptosis on breast cancer, colon cancer and prostate cancer cell lines treated with solanum nigrum extract.

Usually cells undergoing apoptosis display a very similar pattern of morphological changes. These include blebbing, loss of cell membrane symmetry and attachment, cell shrinkage, nuclear fragmentation and chromatin condensation. As illustrated in figure 6, treatment of SKMEL cells with AgNPS of *Indigofera longeracemosa* exhibited typical features of apoptosis including surface bleb formation, redistribution and compaction of cytoplasmic organelles, formation of cytoplasmic vacuoles and hyper convolution of the nuclear membrane.

**DNA Fragmentation Analysis**

In order to evaluate the apoptotic effect of the AgNPS of *Indigofera longeracemosa,* DNA fragmentation assay was performed with three different concentrations. A typical ladder pattern of inter nucleosomal fragmentation was observed in cells treated with higher concentration of AgNPS of *Indigofera longeracemosa* such as 50 and 100µg/ml (fig 7). The results confirmed the induction of apoptosis in SK-MEL-28 cells treated with AgNPS of *Indigofera longeracemosa*

Similar type of inter nucleosomal DNA fragmentation in Human breast cancer colon carcinoma and prostate cancer cell lines treated with *solanum nigrum* was observed [31].

Apoptosis was a consequence of a highly complicated cascade of cellular events, and caspase-3 has been implicated in the execution phase of apoptosis.

**Expression Analysis of P53 and Bcl2 genes**

RT PCR analysis clearly depicted signficant variation in anti apoptitic gene BcL 2 and tumor suppressor gene p53. Approximately 40% increase in band intensity of p53 gene can be correlated to the regulation of cell cycle (Fig 8). Bcl2 shares a considerable role in regulation of multiple drug resistance and AgNPS of *Indigofera longeracemosa* considerably decreased expression of Bcl2 which can be considered significant. This finding is in agreement with many studies that demonstrated the role of p53 and Bcl-2 in inducing apoptosis [32,33]. Most of the drugs currently used to treat cancer patients exert their anti-tumor activity via p53-dependent tumor suppression. Thus, the development of effective drugs which can reactivate wild-type p53 tumor suppressor function is an attractive therapeutic strategy [34]. The p53 can down regulate Bcl-2 which protects cells from apoptosis. Bcl-2 family members mediate anti-apoptotic signals in a wide variety of human cell systems [33, 35]. The Bcl-2 oncoprotein suppresses or delays the induction of apoptosis in prostate, skin, lymphoid tissues, and mammary gland [36, 37] .

**Table 1. Anticancer activity of Biosynthesized silver nanoparticle on SK MEL-28 cell line**

| Sample concentration(µg/ml) | OD at 540nm | % viability |
|---|---|---|
| **Control** | 1.1043 | 100 |
| **Standard drug(sorafenib)** | | |
| 10 | 0.6318 ± 0.57 | 57.21 |
| 50 | 0.5224 ± 0.36 | 47.30 |
| 100 | 0.2229 ± 0.87 | 20.18 |
| **Sample** | | |
| 10 | 0.8303 ± 1.24 | 75.18 |
| 50 | 0.5473 ± 0.65 | 49.56 |
| 100 | 0.4862 ± 0.58 | 44.02 |

**Fig 1. Photograph of a fresh aqueous *Indigofera longeracemosa* extract and Formation of Silver Nanoparticles**

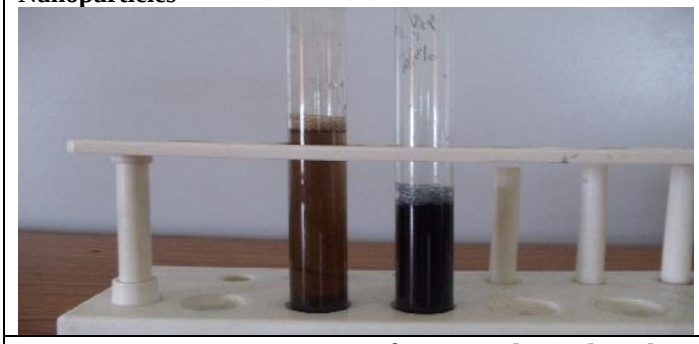


**Fig 2. UV-VIS spectra of Biosynthesized Silver Nanoparticles**

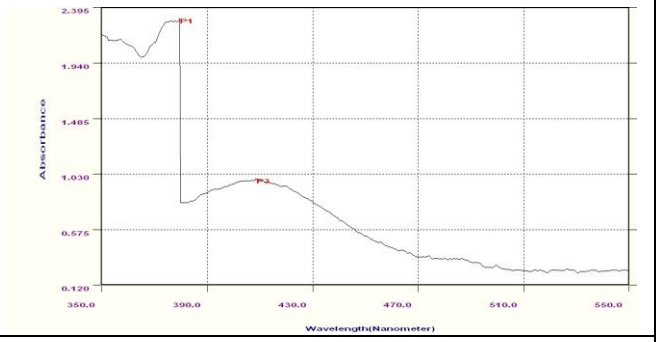



**Fig 3. FT-IR spectrum of Biosynthesized Silver Nanoparticles**

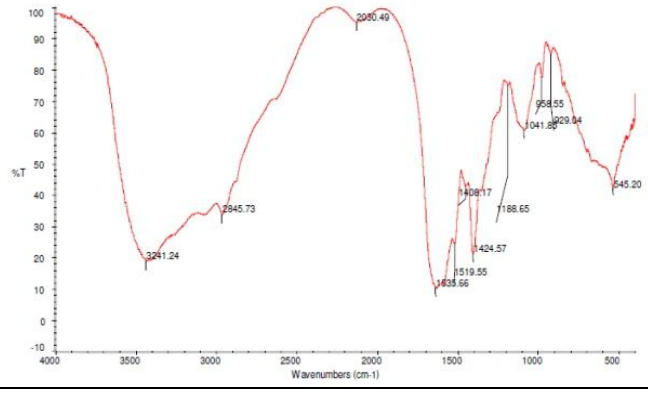



**Fig 4. XRD pattern of Biosynthesized Silver Nanoparticles at 2Ɵ**

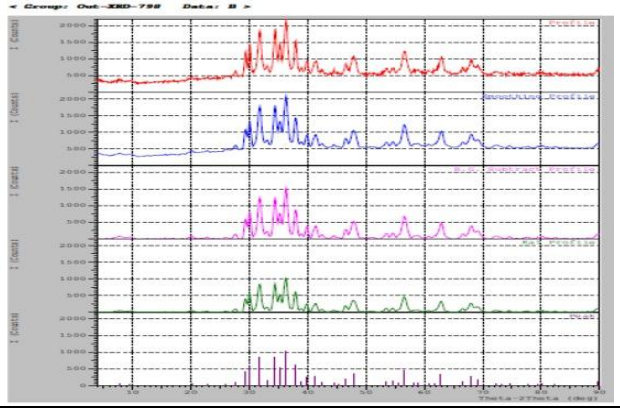


**Fig 5. SEM Micrograph of Biosynthesized Silver Nanoparticles**

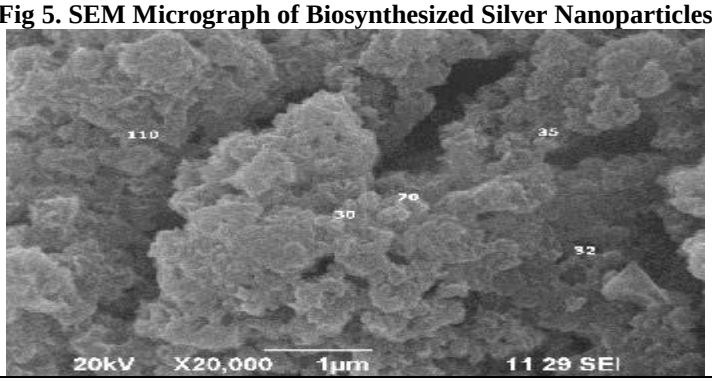
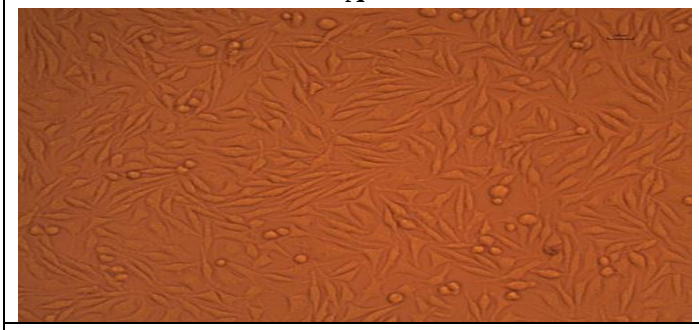
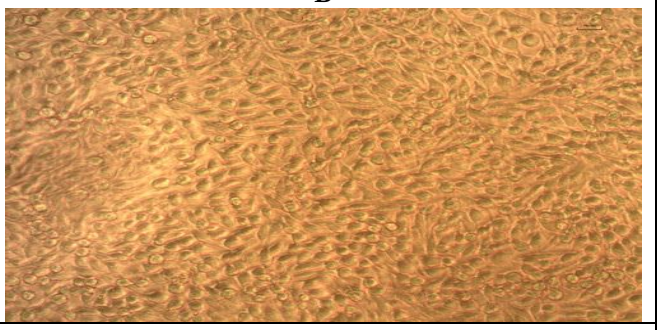



**Fig 6. Dose dependent phase contrast microscopic images of SKMEL 28 cell lines treated with AgNPS of I*ndigofera longeracemosa* as compared to control. [A] Control [B] 10µg/ml [C] 50 µg/ml [D] 100 µg/ml. Photographs were taken using an Olympus camera at a magnification of 40X.**

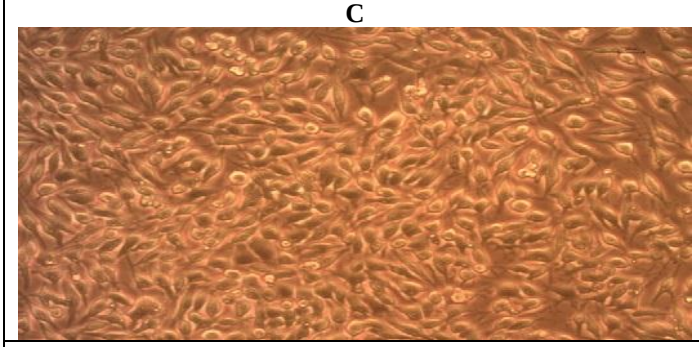
**A**



**B**



**C**



**D**

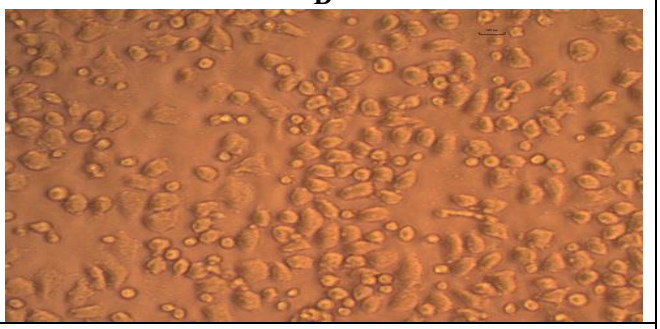
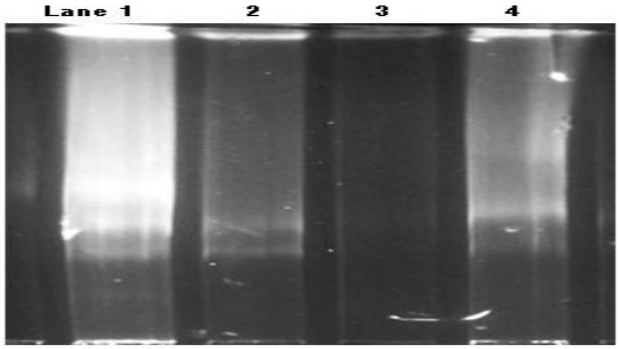
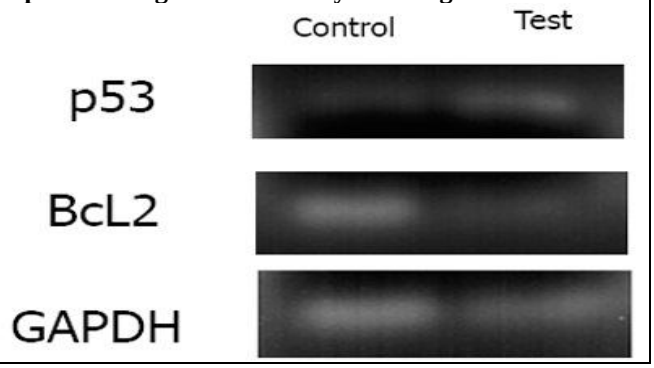


**Fig 7. Induction of apoptosis in SK-Mel-28 cells by various concentrations of AgNPS of *Indigofera longeracemosa***




Lane 1: SK Mel cells treated with 100µg/ml
Lane 2: Untreated control cells
Lane 3: SK Mel cells treated with 10µg/ml
Lane 4: SK Mel cells treated with 50µg/ml

**Fig 8. Effects of $IC_{50}$ concentration of AgNPS of *Indigofera longeracemosa* on the mRNA levels of p53 and Bcl-2 in T47D cells. After 24 hrs incubation with $IC_{50}$ concentrations of extract, total RNA was isolated from treated and control samples and alteration in the expression of genes were analyzed using RT-PCR**



## CONCLUSION

The development of reliable and ecofriendly process for the synthesis of metallic nanoparticles is of great importance in the field of nanotechnology. The present biosynthesis method is a green low cost approach, capable of producing silver-nanoparticles in laboratory ambience. The synthesis of AgNPs might have resulted due to different metabolites (like organic acids, fatty acids and quinones) or metabolic fluxes and other oxido-reductively labile metabolites like ascorbates or catechol/protocatacheuic acid along with different alkaloids. The biosynthesised nanoparticles have been characterized by SEM, FT-IR, XRD and UV-VIS spectroscopy. The AgNPs are crystalline in nature and the size of silver nanoparticles is ranging from 30nm to 110nm**.** Cell morphology, cell viability, DNA fragmentation and expression of tumor suppressor gene were analyzed using AgNPS of *Indigofera longeracemosa*

The biologically synthesised AgNPs showed a considerable cytotoxic effect on SK MEL-28 cell line with an $IC_{50}$ value of 48µg/ml. Morphological analysis of SKMEL 28 cells with AgNps of *Indigofera longeracemosa*at different concentrations (10, 50 and 100 µg/ml) showed typical apoptosis characterized by chromatin condensation and cytoplasmic vacuolization. DNA fragmentation assay confirmed the induction of apoptosis in SK-MEL-28 cells treated with AgNPS of *Indigofera longeracemosa.* Apoptotic cells were characterized by condensed and fragmented DNA. From RT PCR analysis it was clear that, AgNPS of *Indigofera longeracemosa* decreased the expression of anti apoptitic gene BcL 2 and increased the expression of tumor suppressor gene p53.Since the AgNPS of *Indigofera longeracemosa* observed to be the most effective in cell growth inhibition and induction of apoptosis in SK-MEL-28 cells, it can be considered as effective cytotoxic agent. Further toxicity studies of AgNPS of *Indigofera longeracemosa* on animal models might open a door for a new range of anticancer agents.

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